Abortifacient Activity in Beef Cattle of Acetyl- and Succinylisocupressic Acid from Ponderosa Pine

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Acetyl- and succinylisocupressic acids were prepared by derivatization of isocupressic acid isolated from ponderosa pine. The isocupressic acid derivatives were tested and found to induce abortions in late gestation cows after oral dosage starting on day 250 of gestation. Incubation of acetyl- and succinylisocupressic acid in bovine ruminal fluid resulted in hydrolysis of the two esters and the production of isocupressic acid. The abortifacient activity of the isocupressic acid derivatives was thus attributed to their ruminal conversion and production of isocupressic acid.

Keywords: *Pinus ponderosa; premature partuition; bovine abortion; ruminal ester hydrolysis*

INTRODUCTION

Recently we reported on the activity of isocupressic acid (**1**; Figure 1), isolated from ponderosa pine (*Pinus ponderosa*) needles, as an abortifacient compound in beef cattle (Gardner et al., 1994). Late gestation abortions in cattle after consumption of ponderosa pine needles were demonstrated experimentally in the early 1950s (MacDonald, 1952), and the pine needle dose–response relationships were established by James et al. (1977, 1989). James et al. (1994) later demonstrated that the abortifacient activity of the pine needles could be removed by extraction of the ground pine needles with methylene chloride, and abortions could then be induced by feeding the resulting crude pine needle extracts. After bioassay-guided fractionation of the pine needle extract, isocupressic acid (**1**) was isolated and demonstrated to be an abortifacient constituent of the needles (Gardner et al., 1994).

Isocupressic acid (**1**) was first isolated from *Cupressus sempervirens*, the Mediterranean cypress (Mangoni and Belardini, 1964). We are unaware of any reported cattle abortions associated with *C. sempervirens*. However, there are reports of induced abortions in cattle associated with accidental ingestion of *Cupressus macrocarpa* in New Zealand (MacDonald, 1956; Sloss and Brady, 1983). *C. macrocarpa* samples from New Zealand sites have been analyzed, and isocupressic acid was detected with concentrations as high as 1.4% (Parton et al., 1996). The detection of isocupressic acid (**1**) in *C. macrocarpa* further implicates this compound as an abortifacient in cattle and establishes the probable etiology of *C. macrocarpa*-induced cattle abortions in New Zealand.

The occurrence of isocupressic acid (**1**) and two derivatives, acetylisocupressic acid (**2**) and succinylisocupressic acid (**3**), in ponderosa pine needle resin was reported by Zinkel and Magee (1991). All three *P. ponderosa* varieties (var. scopulorum, var. ponderosa, and var. arizonica) found in the western United States contained compounds **1**–**3** in variable amounts. In our original large scale fractionation of abortifacient compounds from ponderosa pine needles, acetyl- and succinylisocupressic acids were not identified (Gardner et al., 1994), but we speculated at that time that, if present, these simple ester derivatives were converted to isocupressic acid (**1**) by alkaline hydrolysis during the base/acid extraction procedures that were used to separate the crude diterpene resin acid compounds from the pine needle extract. Since both acetyl- and succinylisocupressic acids are susceptible to ester hydrolysis (Zinkel and Magee, 1991), it is likely that they may be converted to the free alcohol **1** in the rumen of the cow. The objectives of the work reported here were to prepare acetyl- and succinylisocupressic acid compounds for testing in feeding trials with cattle for abortifacient activity and, secondly, to measure possible ester hydrolysis of compounds **2** and **3** to isocupressic acid (**1**) in cow rumen inoculum.

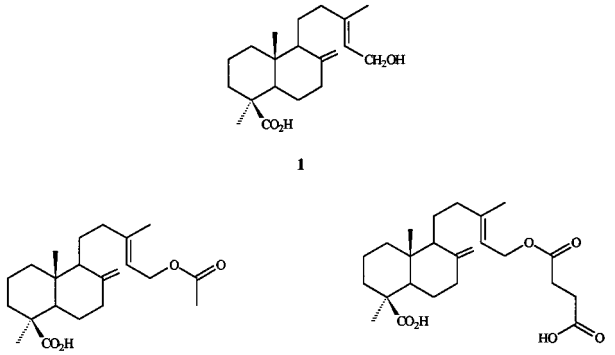

Figure 1. Isocupressic acid (**1**), acetylisocupressic acid (**2**), and succinylisocupressic acid (**3**).

MATERIALS AND METHODS

Extractions and Preparations of Isocupressic Acid Derivatives. Freshly ground ponderosa pine bark (12 kg) was extracted twice by steeping with methylene chloride (30 L) for 48 h. The drained and filtered methylene chloride solutions were concentrated (in vacuo, 55 °C) and stored (5 °C) as a 30–50% (w/v) solution in methylene chloride. Numerous batches were similarly prepared as needed. Figure 2 outlines the preparation of materials to be tested for abortifacient activity.

Phase 1: Preparation of Crude Acetyl- and Succinylisocupressic Acids. Portions of the ponderosa pine bark extract

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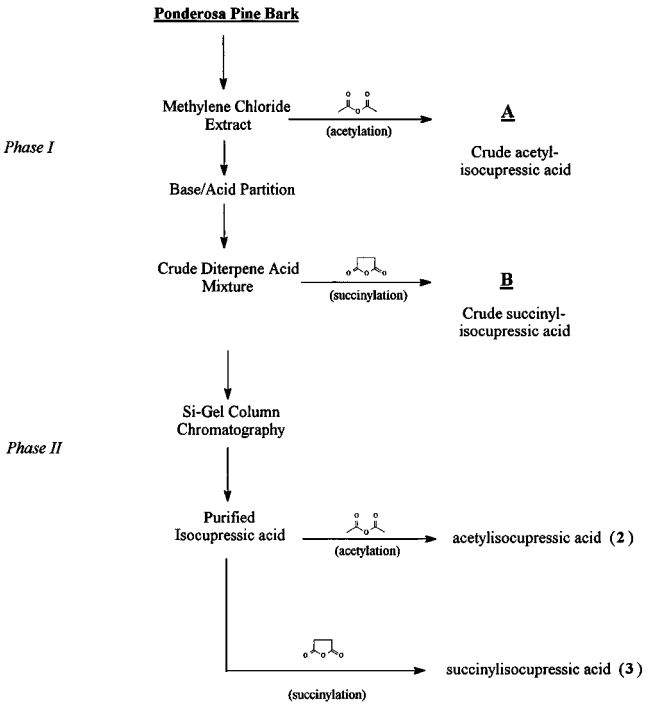

Figure 2. Scheme for preparation of materials tested for abortifacient activity in beef cattle.

(containing approximately 15% isocupressic acid and 35% acetylisocupressic acid) were acetylated as follows. Pine bark extract (340 g) was dissolved in $CHCl_3$ (2.5 L), acetic anhydride (30 g) was added, and the mixture was refluxed for 24 h. The reaction mixture was washed (2 L, H_2O) and the solvent removed by rotary evaporation (in vacuo, 65−80 °C) to yield the final product identified as test material A. The acetylisocupressic acid content of A was approximately 55% by gas chromatography (GC). It is known that the remaining materials (other diterpene resin acids) are not active as abortifacient compounds from previous testing (Gardner et al., 1994).

The crude succinylisocupressic acid was prepared after portions (340 g) of the ponderosa pine bark extract were liquid/liquid extracted using the base/acid scheme previously reported (Gardner et al., 1994). In brief, the crude diterpene resin acid fraction was isolated after extraction of the pine bark methylene chloride extract into aqueous sodium hydroxide solution (0.75 M), the pH was adjusted to 2 with concentrated HCl, the diterpene acids were extracted back into methylene chloride, and the solvent was removed to yield a crude diterpene resin acid fraction. This crude acid fraction (280 g) was dissolved in $CHCl_3$ (2 L), succinic anhydride (75 g) was added, and the mixture was refluxed for 24 h. The solution was cooled in an ice bath for several hours and filtered to remove unreacted succinic anhydride. The solvent was removed by rotary evaporation (in vacuo, 65 °C) to yield test material B containing succinylisocupressic acid (**3**) (65% by GC).

Phase 2: Preparation of Purified Acetyl- and Succinylisocupressic Acids. Isocupressic acid (**1**) was isolated after column chromatography of the crude diterpene acid mixture over silica gel as previously described (Gardner et al., 1994). Isocupressic acid (200 g) was dissolved in $CHCl_3$ (2 L), acetic anhydride or succinic anhydride was added (1.5 mol equiv), and the mixture was refluxed for 16 h. The solvent was removed by rotary evaporation (in vacuo, 65−80 °C). Acetyl- and succinylisocupressic acid content was at least 85%, as assayed by GC (O-TMS derivative), with less than 1% isocupressic acid remaining.

Identification of the two compounds was confirmed by GC/MS analysis of the methylated derivative and comparison to literature values (Fujii and Zinkel, 1984; Zinkel and Magee, 1991). GC/MS data were obtained using a Finnigan MAT GCQ system. Electron ionization (EI) spectra were obtained at 70 eV and a source temperature of 150 °C. Ammonia was used as the reagent gas for chemical ionization. Sample introduction was via the coupled GC using splitless injection.

Table 1. Results of Feeding Trials with Acetyl- and Succinylisocupressic Acids for Abortifacient Activity in Cattle

treatment	dose[a]	no. of abortions	parturition[b]
control		0/4	28, 30, 34, 31
A, acetyl-ICA (crude)	210	1/1	3
B, succinyl-ICA (crude)	250	1/1	3
acetyl-ICA (purified)	120	2/2	5, 6
succinyl-ICA (purified)	138	2/3	5, 30, 6

[a] Dose = (mg/kg) twice per day. [b] Parturition = days from start of treatment to parturition. ICA = isocupressic acid.

Methyl acetylisocupressate: EIMS *m/z* (% relative abundance) 361 (4), 316 (38), 301 (42), 257 (85), 241 (100), 189 (55), 121 (92); CIMS (NH_3) 394 (12, $[M + NH_4]^+$), 377 (5, $[M + H]^+$), 334 (35, $M^+ - COCH_2$), 317 (100, $M^+ - CO_2CH_3$).

Dimethyl succinylisocupressate: EIMS *m/z* (% relative abundance) 316 (25), 301 (36), 257 (100), 241 (86); CIMS (NH_3) 466 (11, $[M + NH_4]^+$), 449 (1, $[M + H]^+$), 354 (8), 334 ($M^+ - COCHCH_2CO_2CH_3$), 317 (100, $M^+ - CO_2C_2H_4CO_2CH_3$), 257 (21), 150 (50).

Animal-Feeding Trials. The protocol for animal use in this research was reviewed and approved by the Institutional Animal Care and Use Committee (IACUC), Utah State University, Logan. Pregnant beef cows (average weight 450 kg) with known breeding dates were used to test the abortifacient activity of the prepared acetyl- and succinylisocupressic acid materials. Animals were treated as previously described (Gardner et al., 1994) with test materials mixed with alfalfa hay (0.45 kg) and administered by gavage (hand pumped with stomach tube) to pregnant cows, starting on days 250−252 of pregnancy, in 2 doses/day (morning and afternoon) for up to 10 days. Treatments are summarized in Table 1, and dosages are listed as mg of test material/kg of animal body weight. The induced parturition was considered to be an abortion if it occurred before 270 days of gestation, and fetal membranes were retained for more than 12 h (Gardner et al., 1994).

Treatment doses were prepared by mixing approximately 125 mL (1.4 M solution in methylene chloride) of the acetyl- and succinylisocupressic acid with 0.45 kg of alfalfa hay. Residual solvent was removed by air-drying in a fume hood for at least 2 h with frequent mixing. Control doses consisted of 0.45 kg of alfalfa hay containing 8 g of acetic anhydride or 4 g of succinic anhydride. These are the estimated theoretical maximum or less of unreacted anhydrides per dose. Actual doses contained very little unreacted anhydrides as they were removed by extraction with water, in vacuo evaporation (acetic anhydride and acetic acid), and filtration (succinic anhydride).

In Vitro Ruminal Digestion of Acetyl- and Succinylisocupressic Acids. In vitro ruminal digestion flasks were prepared with modifications to the Tilley and Terry technique for the digestion of forage crops (Tilley and Terry, 1963; Harris, 1967). Ruminally cannulated donor animals were maintained on a diet of alfalfa hay and taken off of feed and water 1 h before ruminal fluid collection. Strained ruminal fluid inoculum (200 mL) was combined with 800 mL of McDougall's buffer maintained at 39 °C under anaerobic conditions. Ruminal solution (250 mL) was placed in a 300 mL Erlenmeyer flask, the head space of the flask was filled with CO_2, and the flask was stoppered with a Bunsen valve and maintained at 39 °C in a water bath. Ground alfalfa hay (500 mg) was added to the flask and the flask flushed with CO_2 before replacing the Bunsen valve. The mixture was allowed to digest for 24 h, and then 500 mg of treated ground alfalfa hay containing the test material was added to the rumen mixture. Treated alfalfa hay (500 mg) contained 0.16 mmol of isocupressic, acetylisocupressic, or succinylisocupressic acid.

Aliquots (5.0 mL) from the digestion flasks were removed for each treatment approximately every 0.5 h (exact time period of collections was recorded), and rumen activity was stopped by adding concentrated HCl dropwise to pH 2. The sample was extracted with 5 mL of $CHCl_3$. The $CHCl_3$ solution

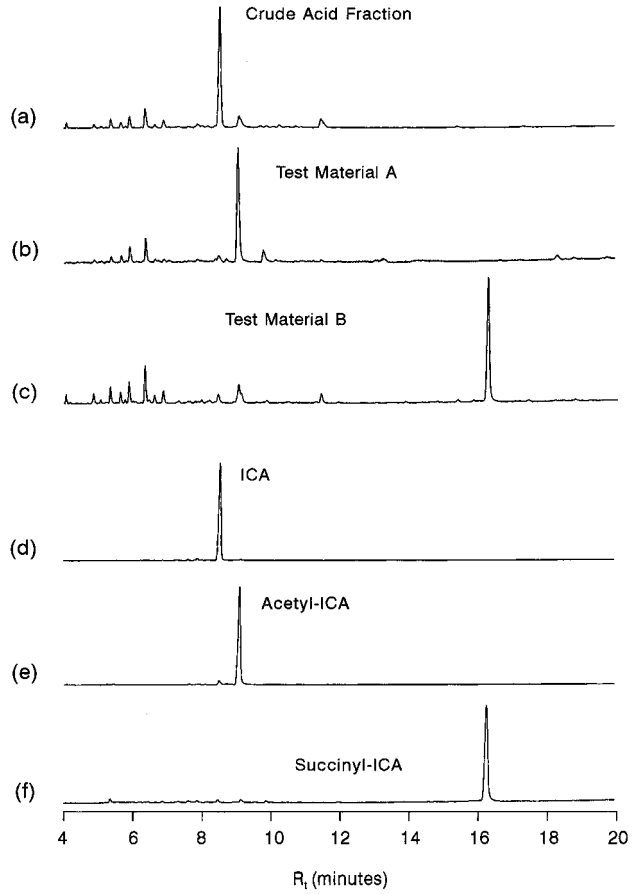

Figure 3. GC chromatograms (O-TMS derivatives) showing (a) isolated crude acid fraction from ponderosa pine bark, (b) test material A, the crude acetylisocupressic acid, (c) test material B, the crude succinylisocupressic acid, (d) isocupressic acid after purification by column chromatography, (e) purified acetylisocupressic acid, and (f) purified succinylisocupressic acid. ICA = isocupressic acid.

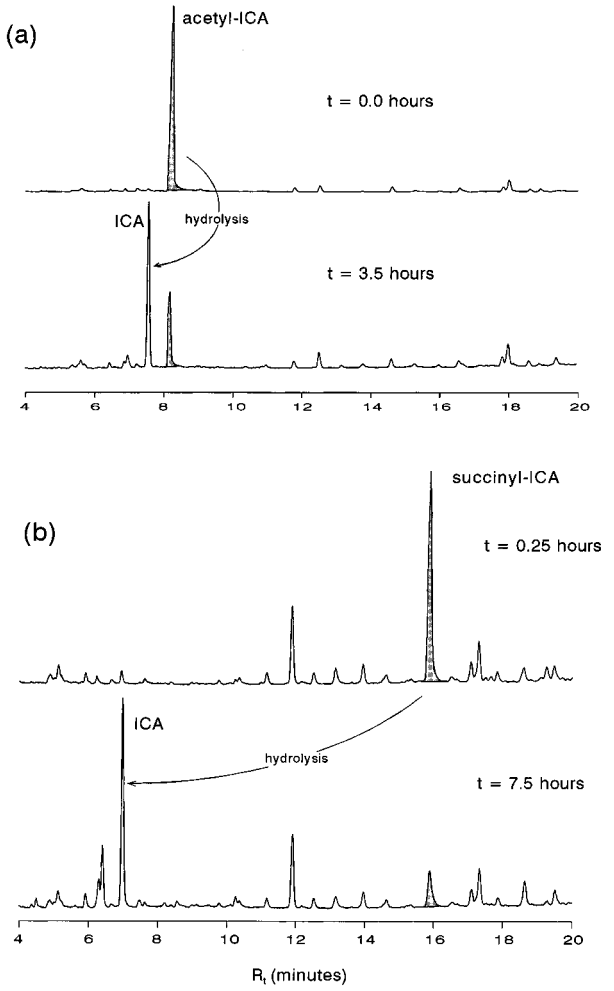

Figure 4. (a) GC chromatograms (O-TMS derivatives) of samples taken from in vitro rumen digestion of acetylisocupressic acid. (b) GC chromatograms (O-TMS derivatives) of two samples taken from in vitro rumen digestion of succinylisocupressic acid. ICA = isocupressic acid.

was filtered through anhydrous Na_2SO_4 and the solvent evaporated under nitrogen at 60 °C. Samples were dissolved in 1.0 mL of pyridine and silylated using 50 μL of BSTFA (Pierce Chemical) reagent in preparation for analysis by GC.

Isocupressic acid and the acetyl and succinyl derivatives were analyzed by GC using a Hewlett-Packard HP 5890 II gas chromatograph equipped with on-column injection and a DB-5 (J&W Scientific) capillary column (15 m × 0.32 mm i.d.) using helium carrier gas at a constant pressure of 8.0 psi. Oven temperature was programmed starting at 100 °C for 0.1 min, 100−200 at 50 °C/min, and 200−300 at 5 °C/min. Detector (FID) temperature was 325 °C.

RESULTS

Feeding Trials with Acetyl- and Succinylisocupressic Acids. *Phase 1 Treatments.* Phase 1 trials proceeded with the preparation of crude extracts from ponderosa pine bark in which all isocupressic acid was converted to either the acetyl or the succinyl derivative. These two preparations were identified as test materials A and B as outlined in Figure 2. Figure 3b,c displays the chromatograms after analysis of A and B by GC. These preparations of acetyl- and succinylisocupressic acids were mixed with ground alfalfa hay, and each was tested by oral dosage in one pregnant cow starting on day 250 of gestation. This assay procedure was identical with that previously used for isocupressic acid (Gardner et al., 1994). Both cows given doses of materials A and B aborted calves after 3 days (Table 1).

Phase 2 Treatments. Isocupressic acid was isolated and purified using column chromatography (Figure 3d) and then derivatized to yield the purified acetylisocupressic acid (Figure 3e) and succinylisocupressic acid (Figure 3f). Feeding trials with the purified isocupressic acid derivatives confirmed their abortifacient activity in cattle. Cows aborted after 5 and 6 days from the start of treatment with acetylisocupressic acid. One of the initial two cows treated with succinylisocupressic acid aborted after 5 days. Subsequently a third cow was treated with succinylisocupressic acid, and this cow also aborted after 6 days of treatment (Table 1). None of the control animals aborted calves.

In Vitro Ruminal Digestion of Acetyl- and Succinylisocupressic Acids. A simple in vitro test was designed to monitor ester hydrolysis of **2** and **3** in the rumen. Small doses of hay containing either acetyl- or succinylisocupressic acid were added to prepared in vitro rumen digestion flasks and allowed to incubate over approximately an 8 h period. Samples from the incubation flasks were collected periodically during the day and the aliquots analyzed for isocupressic acid and its derivatives by gas chromatography (Figure 4). The relative amount of isocupressic acid was calculated, recorded, and plotted versus time (Figure 5). One sample was prepared containing only isocupressic acid. No changes in isocupressic acid level were observed in this control sample over an 8 h period. In the case of

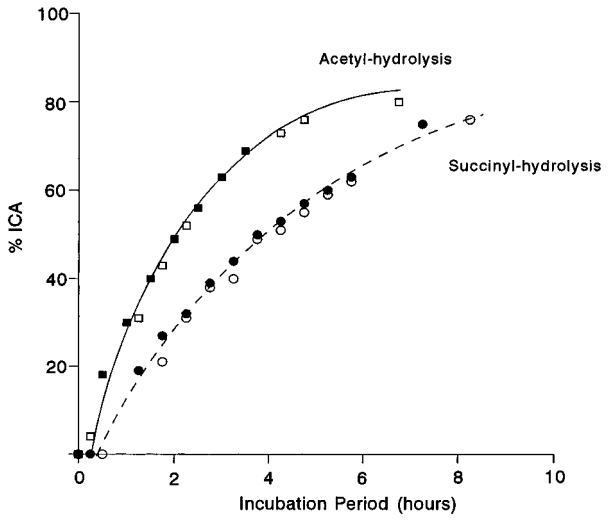

Figure 5. In vitro rumen hydrolysis of acetyl- and succinyl-isocupressic acids. % ICA = relative percent of isocupressic acid versus total isocupressic acid and acetyl- or succinylisocupressic acid. Solid and open symbols represent the two sets of samples run for each experiment.

isocupressic acid derivatives, acetylisocupressic acid was ruminally converted to isocupressic acid with 50% hydrolysis after 2 h. Succinylisocupressic acid was also ruminally converted to isocupressic acid, with 50% hydrolysis occurring after approximately 4 h.

DISCUSSION

It is well documented that ponderosa pine needles induce abortion in late gestation cattle after consumption of the needles (James et al., 1989). Our previous work identified isocupressic acid as an abortifacient component of the pine needles after isolation and feeding trials with this compound (Gardner et al., 1994). However, both acetylisocupressic acid (**2**) and succinyl-isocupressic acid (**3**) are also major components of ponderosa pine needle resin (Zinkel and Magee, 1991). In fact, the amount of isocupressic acid (**1**) that we originally isolated (Gardner et al., 1994) was most likely a combined total of isocupressic acid and hydrolyzed acetyl- (**2**) and/or (**3**) succinyl-isocupressic acid compounds from the plant material. Analysis of ponderosa pine needles using procedures to avoid the ester hydrolysis confirmed the presence of acetylisocupressic acid in our original collection of pine needles and bark (unpublished data). Succinylisocupressic acid was not detected, but its occurrence in other samples is well documented (Zinkel and Magee, 1991). The preponderance of acetyl- and succinylisocupressic acids in plant material known to induce abortions in cattle (Panter et al., 1990) led us to conclude that these esters of isocupressic acids may well be active abortifacient compounds in cattle, either independently or as sources of total available isocupressic acid.

In tests presented here using late gestation pregnant cows, we found that the two derivatives of isocupressic acid are abortifacients from the pine needles. Both acetylisocupressic acid (**2**) and succinylisocupressic acid (**3**) induced abortions in cattle with clinical signs identical with abortions induced by either pine needles or isocupressic acid. We propose that the abortifacient activity of the two natural isocupressic acid derivatives results after ester hydrolysis in the rumen of the cow, thus producing isocupressic acid (**1**). This hypothesis was verified upon in vitro incubation of acetyl- and succinylisocupressic acid in active cow ruminal fluid where both esters are rapidly converted to the free alcohol **1** (Figure 4).

The mechanism by which isocupressic acid induces abortions in cattle is not known. It has been proposed that ponderosa pine needles induce the abortion in cows through vasoconstriction of the caruncular arteries decreasing uterine blood flow (Ford et al., 1992; Christenson et al., 1993). The connection between isocupressic acid and vasoactivity is not evident. Using an in vitro placentome preparation to assay vasoactive compounds, Al-Mahmoud et al. (1995) identified a group of alkanediols esterified with myristic and lauric acids as vasoactive compounds from ponderosa pine needles. Diterpene acids, such as isocupressic acid, were not found in any of the vasoactive fractions (Al-Mahmoud et al., 1995). Postruminal absorption and metabolism of isocupressic acid may be important factors needed to understand the physiological bases for the induced abortions in cattle after ingestion of isocupressic acid or its naturally occurring acetyl and succinyl derivatives.

The economic impact of pine needle abortion is estimated to be between $5 and $20 million annually for Western livestock producers (Lacey et al., 1988; Miner et al., 1987). Understanding the cause and mechanism of the abortions is an important step in developing proper management strategies to reduce the economic losses attributed to pine needle abortion. The implications of the current work are (1) in assessing the potential abortifacient activity of ponderosa pine needles, one needs to account for the total available isocupressic acid in the plant material, and (2) if ester hydrolysis of the isocupressic acid derivatives in the rumen of the cow could be reduced or eliminated, the abortifacient potency of pine needles might be reduced.

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